PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BOARD OF PATENT APPEALS AND INTERFERENCES

CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this APPEAL BRIEF and the documents referred to as enclosed therein are being deposited with the United States Postal Service on the date indicated below with sufficient postage as First Class Mail in an envelope addressed to: Mail Stop Appeal Brief-Patents, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

2/9/06
Date of Deposit

Bethany Crandell

Applicant: Sherman	
Serial No.: 09/277,064	Group Art Unit: 1642
Filed: March 26, 1999	Confirmation No.: 3058
Title: *IN VIVO* ACTIVATION OF TUMOR-SPECIFIC CYTOTOXIC T CELLS	Examiner: M. Davis
	Our Ref.: TSRI 433.1 D1

APPEAL BRIEF

Mail Stop Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

The Applicant appeals the Final Office Action, dated February 9, 2005, and the rejection of claims 1 and 61-75.

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01 FC:1402 500.00 OP
~~02 FC:1254 1590.00 OP~~

Real Party in Interest

The present application has been assigned by the inventor to The Scripps Research Institute, which is the real party in interest.

Related Appeals and Interferences

There are no related appeals or interferences.

Status of Claims

Claims 1 and 61-75 are pending.

Claims 2-60 are canceled.

Claims 1 and 61-75 are rejected.

Claims 1 and 61-75 are on appeal.

Status of Amendments

A Response mailed August 9, 2005 was submitted in reply to the Final Office Action mailed February 9, 2005. However, no Advisory Action was issued in reply to the Response to the Final Office Action. Appellant respectfully submits that the issuance of an Advisory Action by the Examiner is required (see, e.g., MPEP 8th Edition, Revision 3 § 706.07(f)(III)(I) and § 714.13 (III)) and Appellant requests that prosecution on the merits be reopened so that Appellant's Response mailed August 9, 2005 can be considered by the Examiner because Appellant believes that said Response places the claims in condition for allowance. In the event that examination on the merits is not reopened, then the claim amendments set forth in the Response dated August 9, 2005 were not entered into the record, and the arguments are entered into the record. All other amendments in the prosecution history are entered into the record.

Summary of Claimed Subject Matter

Appellant's invention is directed to a method of specifically activating cytotoxic T lymphocytes (CTLs) *in vivo* in an animal having malignant cells that express a Her-2/neu protein, described at specification page 5, line 8, lines 11-14, and lines 24-27 and page 41, lines 24-27; and a method of treating a patient having a tumor wherein cells of the tumor express a Her-2/neu protein, described at specification page 5, line 8; page 7, lines 1-3 and lines 9-12 and page 41, lines 11-14. The claims on appeal are set forth in the Appendix.

Grounds of Rejection to be Reviewed on Appeal

Issue 1

Are claims 1 and 61-75 enabled under 35 U.S.C. § 112, first paragraph?

Grouping of Claims

Issue 1

Claims 1 and 69-75 stand or fall together with respect to Issue 1.

Claims 61-68 stand or fall together with respect to Issue 1.

Arguments

Grouping of Claims for Issue 1

Claims 1 and 69-75 are related to a method of specifically activating cytotoxic T lymphocytes (CTLs) *in vivo* in an animal having malignant cells that express a Her-2/neu protein. The Examiner has rejected claims 1 and 69-75 under Issue 1, by alleging that specific activation of CTLs *in vivo* in an animal having malignant cells that express a Her-2/neu protein is not predictable. Claims 61-68 do not include the element of specifically activating CTLs in such an animal. Therefore, claims 1 and 69-75 stand or fall independently of claims 61-68 with respect to Issue 1.

Claims 61-68 are related to a method of treating a patient having a tumor expressing a Her-2/neu protein. The Examiner has rejected claims 61-68 under Issue 1 by alleging that treating a patient having a tumor expressing a Her-2/neu protein is unpredictable. Claims 1 and 69-75 do not include the element of treating a patient having a tumor expressing a Her-2/neu protein. Therefore, claims 61-68 stand or fall independently of claims 1 and 69-75 with respect to Issue 1.

Issue 1

Rejection under 35 U.S.C. § 112, first paragraph:

Claims 1 and 61-75 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly not being enabled for several reasons as discussed below. Appellant respectfully traverses this rejection as discussed below.

On page 2 of the Final Office Action mailed February 9, 2005 (hereinafter, the "Final Office Action"), the Examiner asserts that claims 61-75 are rejected allegedly as lacking enablement

for the same reasons as claim 1. However, as discussed in the section above, claims 1 and 69-75 stand or fall independently of claims 61-68 with regard to Issue 1. Therefore, Appellant will first discuss the reasons why the enablement rejection should be withdrawn with respect to claims 1 and 69-75. Then, Appellant will discuss the reasons why the enablement rejection should be withdrawn with respect to claims 61-68.

Claims 1 and 69-75

In the Final Office Action, the Examiner rejected claims 1 and 69-75 for reasons of record in the Office Action mailed July 12, 2004. Appellant respectfully traverses this rejection for reasons which follow and for reasons on record including the those set forth in the Responses filed November 12, 2004, pages 5-13 and August 9, 2005, pages 8-13.

On page 3 of the Final Office Action, the Examiner asserts that the present claims allegedly are not enabled because one skilled in the art allegedly would be forced into undue experimentation to practice the claimed invention because:

1. killing target malignant cells by T cells specific for the polypeptide of SEQ ID NO:12 in patients with cancer burden and having Her-2/neu as self antigen is allegedly unpredictable,

2. there allegedly is a lack of correlation between production of specific CTLs in a transgenic mouse that is cancer free and does not express the particular human HER-2/neu polypeptide of SEQ ID NO:12 as self antigen and the production of CTLs that could kill target cancer

cells in patients having cancer burden and having Her-2/neu as self antigen,

3. there allegedly is a lack of correlation between killing cancer cells *in vitro* and killing primary cancer cells *in vivo*, and

4. there allegedly is a lack of adequate disclosure in the specification in view of the allegedly complex nature of the claimed invention and allegedly little is known in the art about the claimed invention.

The test of enablement is whether one reasonably skilled in the art could make and use the claimed invention from the disclosures in the patent coupled with information known in the art without undue experimentation (see, e.g., *In re Wands*, 858 F.2d at 737,8 USPQ2d at 1404 (Fed. Cir. 1988)).

Claim 1 and dependent claims 69-75 are related to a method of specifically activating cytotoxic T lymphocytes (CTLs) *in vivo* in an animal having malignant cells that express a Her-2/neu protein, comprising immunizing the animal with the polypeptide of SEQ ID NO:12.

Appellant respectfully submits that one reasonably skilled in the art was able to make and use the claimed invention in view of the disclosures of the specification and knowledge available in the art without undue experimentation as discussed on the record and in the points below.

I. The making of the polypeptide of SEQ ID NO:12 is described in the specification, for example, at page 74, lines 2-4 and page 84, lines 26-37.

II. The use of the polypeptide of SEQ ID NO:12 comprising immunizing an animal with said polypeptide is described in the specification, for example, at page 13, lines 11-13, page 79, lines 18-21, page 84, lines 23-24, page 86, lines 36-37, and page 87, lines 24-25.

III. The specific activation of CTLs *in vivo* is described in the specification, for example, at page 105, line 32 through page 106, line 2 using a model system for assaying CTLs that are specifically activated against the polypeptide of SEQ ID NO:12.

IV. Appellant respectfully submits Lustgarten et al. (2004) European Journal of Immunology 34:752-761 (previously entered into the record with the Response mailed August 9, 2005 and attached herewith). Lustgarten et al. demonstrate, after the filing date of the present invention, that immunization of Her-2/neu transgenic mice, which express Her-2/neu and develop spontaneous mammary tumors, with the polypeptide of SEQ ID NO:12 (referred to as p773-782 in the reference) results in the specific activation of CTLs (see, e.g., page 753, column 1, line 25, which states, "they contain a T cell repertoire of low avidity"). Accordingly, Lustgarten et al. supports Appellant's assertion that the presently claimed invention was enabled at the time of filing because Lustgarten et al. used similar methods to those disclosed in the present invention.

Accordingly, Appellant respectfully submits that the claimed invention is enabled because the specification describes how to make and use the claimed invention in view of the knowledge available in the art without undue experimentation which is supported by Lustgarten et al and as discussed in items I-IV above.

Turning to the first item in the Examiner's assertions in items 1-4 above, the Examiner asserts that killing target malignant cells by T cells specific for the polypeptide of SEQ ID NO:12 in patients with cancer burden and having Her-2/neu as self antigen is allegedly unpredictable. The present assertion is misplaced and should be withdrawn because the presently claimed invention does not require the feature of killing target malignant cells.

Turning to the second item in the Examiner's assertions in items 1-4 above, the Examiner asserts that allegedly there is a lack of correlation between production of specific CTLs in a transgenic mouse that is cancer free and does not express the particular human HER-2/neu polypeptide of SEQ ID NO:12 as self antigen and the production of CTLs that could kill target cancer cells in patients having cancer burden and having Her-2/neu as self antigen. The present assertion is misplaced and should be withdrawn because the presently claimed invention does not require the feature of killing target cancer cells in patients.

Furthermore, there is no requirement for patentability that a working example be provided in the specification (see, e.g., MPEP 8r1 2164.02). An applicant need not have actually reduced

the invention to practice prior to filing. *In Gould v. Quigg*, 822 F.2d 1074, 1078, 3 USPQ 2d 1302, 1304 (Fed. Cir. 1987).

Still further, the working examples provided in the specification (see, for instance, Examples 2, 3, and 5 starting on pages 84, 88, and 101, respectively) adequately correlate with the claimed invention as discussed above in items I-IV.

Turning to the third item in the Examiner's assertions in items 1-4 above, the Examiner asserts that allegedly there is a lack of correlation between killing cancer cells *in vitro* and killing primary cancer cells *in vivo*. The present assertion is misplaced and should be withdrawn because the presently claimed invention does not require the feature of killing primary cancer cells *in vivo*.

Turning to the fourth item in the Examiner's assertions in items 1-4 above, the Examiner asserts that allegedly there is a lack of adequate disclosure in the specification in view of the allegedly complex nature of the claimed invention and allegedly little is known in the art about the claimed invention. The present assertion is unsupported by the Examiner and, therefore, should be withdrawn.

Next, the Examiner asserts that the claims allegedly are not enabled because allegedly the nature of the invention and the art is unpredictable as set forth in items A-F of the Final Office Action (see, pages 4-12 of the Final Office Action).

A. Breath of the Claims and Nature of the Invention

On page 4 of the Final Office Action, the Examiner asserts that the scope of the claims is allegedly overly broad,

encompassing a method for treating or specifically activating CTLs in cancer patients with tumor burden, and having Her-2/neu comprising the polypeptide of SEQ ID NO:12 as self-antigen, wherein said CTLs specifically kill target malignant cells that express a Her-2/neu protein of said patients, comprising administering the polypeptide of SEQ ID NO:12.

Appellant wishes to clarify the record in that the presently claimed invention is not related to treating CTLs. Appellant further wishes to clarify the record in that the present claims do not require the polypeptide of SEQ ID NO:12 as self-antigen because the claims are related to an animal having malignant cells that express a Her-2/neu protein. Appellant further requests that the present assertion be withdrawn because the claims to not require the feature of specifically killing target malignant cells.

Next, on page 4 of the Final Office Action, the Examiner asserts that the specification allegedly only discloses activating CTLs in a transgenic mouse that is cancer free. Appellant respectfully submits that a working example is not required, as discussed above. Also, there is no prerequisite for meeting the enablement requirement that the claimed method be tested in a animal having a Her-2/neu tumor as supported by the Federal Circuit which stated:

> "If applicants were required to wait until an animal naturally developed this specific tumor before testing the effectiveness of a compound against the tumor *in vivo*, as would be implied from the Commissioner's argument, there would be no effective way to test

compounds *in vivo* on a large scale". See *In re Brana* at 1440.

Furthermore, MPEP 8th Edition, Revision 3 § 2164.02 states that, "An *in vitro* or *in vivo* animal model example in the specification, in effect, constitutes a 'working example' if that example 'correlates' with a disclosed or claimed method invention". Appellant respectfully submits that the working examples disclosed in the specification, for instance, in Examples 2, 3, and 5 starting on pages 84, 88, and 101, respectively, correlate with the claimed invention for the following reasons. The working examples clearly demonstrate that immunization of an animal with the polypeptide of SEQ ID NO:12 results in the specific activation of CTLs. Also, Lustgarten et al. demonstrate specific activation of CTLs in mice having malignant cells that express a Her-2/neu protein, comprising immunizing the mice with the polypeptide of SEQ ID NO:12, as discussed above.

Next, the Examiner asserts, at the bottom of page 4 of the Final Office Action, that allegedly the polypeptide of SEQ ID NO:12 is of human origin, and the specification allegedly does not disclose that said particular human peptide has the same structure as the corresponding Her-2/neu peptide in transgenic mice. The present allegation is an unsupported assertion and should be withdrawn.

At the top of page 5 of the Final Office Action, the Examiner asserts "the specification only discloses that said cytotoxic T lymphocytes could kill cancer cells line expressing Her-2/neu '*in vitro*', which cannot be correlated with *in vivo* conditions". The present allegation is an unsupported assertion

and should be withdrawn. The present allegation should also be withdrawn because the claims do not require that said CTLs kill cancer cells.

B. The State of the Art and the Level of Skill in the Art

The Examiner asserts on page 5 of the Final Office Action that the step of immunizing with a peptide is routine in the art, and that it is not known in the art that the polypeptide of SEQ ID NO:12 could specifically activate CTLs in an animal having malignant cells that express a Her-2/neu protein, wherein said CTLs specifically kill target malignant cells that express a Her-2/neu protein. The present rejection should be withdrawn because the instant claims do not require that said CTLs specifically kill target malignant cells. Furthermore, Lustgarten et al. demonstrates, after the filing date of the present invention, the specific activation of CTLs by the polypeptide of SEQ ID NO:12 in an animal having malignant cells that express a Her-2/neu protein as claimed. Accordingly, the present rejection should be withdrawn for reasons of record and the reasons discussed above.

C. The Level of Predictability in the Art

On pages 5-10 of the Final Office Action, the Examiner makes various assertions that the "level of unpredictability is very high in the instant application" (see, page 7, line 10). Appellant stands on Appellant's arguments of record with the additional argument that Lustgarten et al. further demonstrates the enablement of present invention using methods similar to those disclosed in the present specification, as discussed above.

D. The Amount of Direction Provided By the Inventor

The Examiner asserts on pages 10-11 of the Final Office Action that the specification provides insufficient guidance

because the working examples provided allegedly cannot be correlated with successful production of specific T cells in patients with cancer burden, and having Her-2/neu comprising the polypeptide of SEQ ID NO: 12 as self antigen, wherein said T cells could successfully kill malignant cells in said patients. Appellant respectfully submits that adequate guidance for the claimed in invention was provided in the specification to enable the claimed invention for reasons of record and for the reasons stated, herein.

E. and F. The Existence of Working Examples and the Quantity of Experimentation Needed

The Examiner asserts on pages 11 through 12 of the Final Office Action that, although a working example is not always required to demonstrate enablement, allegedly one skilled in the art would be forced into undue experimentation to practice the claimed invention for the following reasons (1-4, below). Applicants comments follow each of the Examiner's reasons.

1) Killing target malignant cells by T cells specific for the polypeptide of SEQ ID NO:12 in patients with cancer burden and having Her-2/neu self-antigen is allegedly unpredictable. Appellant responds that the present claims to not require killing target malignant cells and, therefore, the present rejection should be withdrawn.

2) There is an alleged lack of correlation between production of specific T cells in transgenic mice that are cancer free compared and production of T cells that could kill target cancer cells in patients having cancer burden and having Her-2/neu as self antigen. Appellant responds that the present rejection should be withdrawn

because the production of T cells that could kill target cancer cells is not a requirement of the present claims.

3) There allegedly is a lack of correlation between killing cancer cells *in vitro* and killing primary cancer cells *in vivo*. Appellant responds that the present rejection should be withdrawn because killing primary cancer cells is not a requirement of the present claims.

4) There allegedly is a lack of adequate disclosure in the specification in view of the allegedly complex nature of the invention. Appellant responds that this is an unsupported assertion and Appellant stands on the arguments of record and the arguments herein above that the present specification provides sufficient guidance to make and use the instant invention in view of the knowledge in the art without undue experimentation.

On page 13 of the Final Office Action, the Examiner asserts that claims 61-75 allegedly are drawn to a method for treating a patient having a tumor which allegedly is not enabled. The present assertion should be withdrawn in regard to claims 69-75 because these claims to not recite the limitation of treating a patient having a tumor.

Claims 61-68

In the Final Office Action, the Examiner rejected claims 61-68 for reasons of record in the Office Action mailed July 12, 2004. Appellant respectfully traverses this rejection for reasons as set forth above, for reasons which follow, and for reasons of record including the those set forth in the Responses filed November 12, 2004, pages 5-13 and August 9, 2005, pages 8-13.

On page 3 of the Final Office Action, the Examiner asserts that the present claims allegedly are not enabled because one skilled in the art allegedly would be forced into undue experimentation to practice the claimed invention because:

1. killing target malignant cells by T cells specific for the polypeptide of SEQ ID NO:12 in patients with cancer burden and having Her-2/neu as self antigen is allegedly unpredictable,

2. there allegedly is a lack of correlation between production of specific CTLs in a transgenic mouse that is cancer free and does not express the particular human HER-2/neu polypeptide of SEQ ID NO:12 as self antigen and the production of CTLs that could kill target cancer cells in patients having cancer burden and having Her-2/neu as self antigen,

3. there allegedly is a lack of correlation between killing cancer cells *in vitro* and killing primary cancer cells *in vivo*, and

4. there allegedly is a lack of adequate disclosure in the specification in view of the allegedly complex nature of the claimed invention and allegedly little is known in the art about the claimed invention.

The test of enablement is whether one reasonably skilled in the art could make and use the claimed invention from the disclosures in the patent coupled with information known in the art without undue experimentation (see, e.g., *In re Wands*, 858 F.2d at 737,8 USPQ2d at 1404 (Fed. Cir. 1988)).

Turning to the first item in the Examiner's assertions in items 1-4 above (see page 20 of this Appeal Brief), the Examiner asserts that killing target malignant cells by T cells specific for the polypeptide of SEQ ID NO:12 in patients with cancer burden and having Her-2/neu as self antigen is allegedly unpredictable. The present assertion is misplaced and should be withdrawn because the presently claimed invention does not require the feature of killing target malignant cells. Furthermore, the present assertion is misplaced and should be withdrawn because the presently claimed invention does not require the feature of killing target malignant cells by T cells specific for the polypeptide of SEQ ID NO:12.

Turning to the second item in the Examiner's assertions in items 1-4 above (on page 20), the Examiner asserts that allegedly there is a lack of correlation between production of specific CTLs in a transgenic mouse that is cancer free and does not express the particular human HER-2/neu polypeptide of SEQ ID NO:12 as self antigen and the production of CTLs that could kill target cancer cells in patients having cancer burden and having Her-2/neu as self antigen. The present assertion is misplaced and should be withdrawn because the presently claimed invention does not require the feature of production of CTLs that could kill target cancer cells.

Furthermore, there is no requirement for patentability that a working example be provided in the specification (see, e.g., MPEP 8th Edition, Revision 3 § 2164.02). An applicant need not have actually reduced the invention to practice prior to filing. *In Gould v. Quigg*, 822 F.2d 1074, 1078, 3 USPQ 2d 1302, 1304 (Fed. Cir. 1987).

Still further, the working examples provided in the specification (see, for instance, Examples 2, 3, and 5 starting on pages 84, 88, and 101, respectively) adequately correlate with the claimed invention as discussed herein above.

Turning to the third item in the Examiner's assertions in items 1-4 above (on page 20), the Examiner asserts that allegedly there is a lack of correlation between killing cancer cells *in vitro* and killing primary cancer cells *in vivo*. The present assertion is misplaced and should be withdrawn because the presently claimed invention does not require the feature of killing primary cancer cells *in vivo*.

Turning to the fourth item in the Examiner's assertions in items 1-4 above (on page 20), the Examiner asserts that allegedly there is a lack of adequate disclosure in the specification in view of the allegedly complex nature of the claimed invention and allegedly little is known in the art about the claimed invention. The present assertion is unsupported by the Examiner and, therefore, should be withdrawn.

Next, the Examiner asserts that the claims allegedly are not enabled because allegedly the nature of the invention and the art is unpredictable as set forth in items A-F of the Final Office Action (see, pages 4-12 of the Final Office Action).

A. Breath of the Claims and Nature of the Invention

On page 4 of the Final Office Action, the Examiner asserts that the scope of the claims is overly broad, encompassing a method for treating or specifically activating CTLs in cancer patients with tumor burden, and having Her-2/neu comprising the polypeptide of SEQ ID NO:12 as self-antigen, wherein said CTLs

specifically kill target malignant cells that express a Her-2/neu protein of said patients, comprising administering the polypeptide of SEQ ID NO:12.

Appellant requests that the present assertion be withdrawn because the instantly claimed invention does not require specifically activating CTLs or CTLs that specifically kill target malignant cells.

Next, on page 4 of the Final Office Action, the Examiner asserts that allegedly the specification only discloses activating CTLs in a transgenic mouse that is cancer free. Appellant respectfully submits that the present assertion be withdrawn because the instant claims do not recite activating cytotoxic T lymphocytes with the polypeptide of SEQ ID NO:12.

Next, the Examiner asserts, at the bottom of page 4 of the Final Office Action, that the polypeptide of SEQ ID NO:12 allegedly is of human origin, and that allegedly the specification does not disclose that said particular human peptide has the same structure as the corresponding Her-2/neu peptide in the transgenic mice. The present allegation is an unsupported assertion and should be withdrawn.

At the top of page 5 of the Final Office Action, the Examiner asserts "the specification only discloses that said cytotoxic T lymphocytes could kill cancer cells line expressing Her-2/neu '*in vitro*', which cannot be correlated with *in vivo* conditions". The present allegation is an unsupported assertion and should be withdrawn. The present allegation should also be withdrawn because the claims do not require cytotoxic T lymphocyte action.

B. The State of the Art and the Level of Skill in the Art

The Examiner asserts on page 5 of the Final Office Action that the step of immunizing with a peptide is routine in the art, and that it is not known in the art that the polypeptide of SEQ ID NO:12 specifically activates CTLs in an animal having malignant cells that express a Her-2/neu protein, wherein said CTLs specifically kill target malignant cells that express a Her-2/neu protein. The present rejection should be withdrawn because the instant claims do not require administration of the polypeptide of SEQ ID NO:12 to specifically activate cytotoxic T lymphocytes nor do the instant claims require CTLs that specifically kill target malignant cells that express a Her-2/neu protein.

Furthermore, Lustgarten et al. demonstrate, after the filing date of the present invention, that a method comprising administering the polypeptide of SEQ ID NO:12 to an animal having a tumor that expresses a Her-2/neu protein retarded tumor growth (see, e.g., the abstract). Accordingly, the present rejection should be withdrawn for the reasons of record and the reasons discussed above.

C. The Level of Predictability in the Art

On pages 5-10 of the Final Office Action, the Examiner makes various assertions that the "level of unpredictability is very high in the instant application" (see, page 7, line 10). Appellant stands on Appellant's arguments of record with the additional argument that Lustgarten et al. further demonstrate the enablement of present invention using methods similar to those disclosed in the present specification, as discussed above.

D. The Amount of Direction Provided By the Inventor

The Examiner asserts on pages 10-11 of the Final Office Action that the specification provides insufficient guidance because the working examples provided allegedly cannot be correlated with successful production of specific T cells in patients with cancer burden, and having Her-2/neu comprising the polypeptide of SEQ ID NO: 12 as self antigen, wherein said T cells could successfully kill malignant cells in said patients. Appellant respectfully submits that the present assertion should be withdrawn because the instant claims do not require production of specific T cells or killing malignant cells.

E. and F. The Existence of Working Examples and the Quantity of Experimentation Needed

The Examiner asserts on pages 11 through 12 of the Final Office Action that, although a working example is not always required to demonstrate enablement, allegedly one skilled in the art would be forced into undue experimentation to practice the claimed invention for the following reasons (1-4, below). Appellant's comments follow each of the Examiner's reasons.

1) Killing target malignant cells by T cells specific for the polypeptide of SEQ ID NO:12 in patients with cancer burden and having Her-2/neu self-antigen is allegedly unpredictable. Appellant responds that the present rejection should be withdrawn because the present claims to not require killing target malignant cells or the production of T cells specific for the polypeptide of SEQ ID NO:12.

2) There is an alleged lack of correlation between production of specific T cells in transgenic mice that

are cancer free compared and production of T cells that could kill target cancer cells in patients having cancer burden and having Her-2/neu as self antigen. Appellant responds that the present rejection should be withdrawn because the production of T cells that could kill target cancer cells is not a requirement of the present claims.

3) There allegedly is a lack of correlation between killing cancer cells *in vitro* and killing primary cancer cells *in vivo*. Appellant responds that the present rejection should be withdrawn because killing primary cancer cells is not a requirement of the present claims.

4) There allegedly is a lack of adequate disclosure in the specification in view of the allegedly complex nature of the invention. Appellant responds that this is an unsupported assertion and Appellant stands on the arguments of record and the arguments herein above that the present specification provides sufficient guidance to make and use the instant invention in view of the knowledge in the art without undue experimentation.

On page 13 of the Final Office Action, the Examiner asserts that claims 61-75 allegedly are drawn to a method for treating a patient having a tumor which allegedly is not enabled because tumor allegedly encompasses any swelling, or growth, or enlargement of tissue, wherein said tissue expresses a Her-2/neu protein. The present assertion should be withdrawn in regard to claims 61-68 because the specification states that the instant invention is related to cancer, tumors, neoplasia, etc. which make it clear that the instant claims are related to cancerous tumors (see, e.g., page 5, lines 1-3 of the specification).

Summary

For the reasons of record and for the forgoing reasons, Appellant respectfully requests that the Board of Patent Appeals and Interferences reverse the Examiner's rejections under 35 U.S.C. § 112 with respect to claims 1 and 61-75, and remand this application back to the Examiner for further examination.

If there are any fees associated with this Appeal Brief, the Director is authorized to charge our Deposit Account No. 19-0962.

Feb. 9, 2006
Date

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Claims Appendix

Claim 1 (previously presented): A method of specifically activating cytotoxic T lymphocytes *in vivo* in an animal having malignant cells that express a Her-2/Neu protein, the method comprising the step of immunizing said animal with the polypeptide of SEQ ID NO:12.

Claim 61 (Previously presented): A method of treating a patient having a tumor and in need of treatment of said tumor, wherein cells of said tumor express a Her-2/Neu protein, the method comprising the step of administering a polypeptide having the amino acid sequence VMAGVGSPYV (SEQ ID NO:12) to the patient.

Claim 62 (previously presented): The method of claim 61, wherein said polypeptide is incorporated into a pharmaceutical composition further comprising a pharmaceutically acceptable carrier.

Claim 63 (previously presented): The method of claim 61, wherein said polypeptide is linked to a carrier.

Claim 64 (previously presented): The method of claim 61, wherein said polypeptide is administered to said patient as a homopolymer.

Claim 65 (previously presented): The method of claim 61, further comprising the step of administering a second component to said patient, wherein said

second component primes cytotoxic T lymphocytes (CTLs) for activation.

Claim 66 (previously presented): The method of claim 65, wherein the second component comprises tripalmitoyl-S-glycerylcysteinyl-seryl-serine (P3CSS).

Claim 67 (previously presented): The method of claim 61, further comprising the step of administering a second polypeptide to said animal.

Claim 68 (previously presented): The method of claim 67, wherein the second polypeptide comprises TPPAYRPPNAPIL (SEQ ID NO:9).

Claim 69 (previously presented): The method of claim 1, wherein said polypeptide is incorporated into a pharmaceutical composition further comprising a pharmaceutically acceptable carrier.

Claim 70 (previously presented): The method of claim 1, wherein said polypeptide is linked to a carrier.

Claim 71 (previously presented): The method of claim 1, wherein said polypeptide is administered to said animal as a homopolymer.

Claim 72 (previously presented): The method of claim 1, wherein said immunizing step further comprises administering a second component to said animal,

wherein said second component primes said CTLs for activation.

Claim 73 (previously presented): The method of claim 72, wherein said second component comprises tripalmitoyl-S-glycerylcysteinyl-seryl-serine (P3CSS).

Claim 74 (previously presented): The method of claim 1, wherein the immunizing step further comprises administering a second polypeptide to said animal.

Claim 75 (previously presented): The method of claim 74, wherein the second polypeptide comprises TPPAYRPPNAPIL (SEQ ID NO:9).

Evidence Appendix

Copies of evidence relied upon by Appellant are enclosed herewith.

Related Proceedings Appendix

There are no related appeals or interferences.

The CD8+ T cell repertoire against Her-2/neu antigens in neu transgenic mice is of low avidity with antitumor activity

Joseph Lustgarten, Ana Lucia Dominguez and Camilo Cuadros

Sidney Kimmel Cancer Center, San Diego, USA

The majority of tumor-associated antigens are aberrantly expressed or overexpressed normal gene products. Therefore, mechanisms responsible for self tolerance dampen immune responses against these antigens. To evaluate the effect that tolerance has on the immune responses against tumor antigens, we characterized the CD8+ T cell responses in neu mice. T cell responses against the A2.1/neu p369–377 and p773–782 peptides were evaluated in neu mice that were crossed with A2.1/Kb transgenic mice (A2×neu). Tetramer binding and cytotoxic activity demonstrate that, compared to CTL from A2.1/Kb×FVB wild-type mice (A2×FVB), CD8+ T cells from A2×neu mice were of lower avidity for the peptides. Despite the fact that A2×neu mice are tolerant, multiple immunizations with DC pulsed with the p369–377 or p773–782 peptides in the presence of IL-2 retarded tumor growth in A2×neu mice, and immunizations in combination with the anti-OX40 mAb further enhanced the antitumor response. Taken together, these data indicate that low-avidity T cells for neu antigens persisting in A2×neu mice have the capacity to develop antitumor responses as long as they are provided with efficient costimulation. These results underscore the potential role of low-avidity T cells in antitumor immunity and may offer an important component for vaccination immunotherapies.

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Revised	14/1/04
Accepted	2/2/04

Key words: Tumor immunology / Tolerance / Vaccination / CTL / Antigens/peptides

1 Introduction

The discovery of tumor-associated antigens (TAA) [1, 2] has been an important breakthrough in tumor immunology, because it is possible to devise immunotherapeutic approaches to promote T cell responses against such antigens and induce a protective immunity against neoplastic malignancies [3]. The majority of the currently defined TAA are often overexpressed products of normal cellular genes [4]. As such, these overexpressed proteins pose a significant challenge to the design of effective T cell immunotherapies as self tolerance has to be considered [5]. Based on transgenic mouse models, it is now clear that tolerance is capable of deleting reactive high-avidity T cells against the transgene (self), thereby leading to self tolerance [6]. However, T cell elimination through tolerance is not absolute, since self-specific T cells can be isolated from tolerant hosts [7, 8]. Moreover, it has been demonstrated that low-avidity T cells can be activated, expanded and involved in antitumor responses [9, 10]. Thus, the observation that low-avidity T cells persist *in vivo* and that they can recognize self antigens underscores their potential role in antitumor immunity.

The significance of understanding the mechanism responsible for the persistence of low-avidity T cells relates not only to our understanding of autoimmunity, but also to the use of such cells to target self tumor antigens for tumor destruction. Therefore, a central question is whether the available repertoire of T cells specific for up-regulated self tumor antigens is sufficient in number or avidity to mount an effective antitumor response. We have addressed this fundamental question in an experimental model in which the rat *neu* protooncogene is expressed in the mammary tissue under the control of the MMTV promoter (FVB-MMTV-*neu* transgenic mice) [11, 12]. Neu mice develop spontaneous mammary tumors, and the multi-step process for the development of mammary tumors in these animals recapitulates the clinical progression and pathogenesis of the human disease. Thus, this model is ideal for the evaluation of immunological responses against neu antigens as well as the evaluation of novel immunotherapeutic modalities for tumor eradication.

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Abbreviation: TAA: Tumor-associated antigen

Advances in understanding how T cells become activated have led to the development of new strategies to enhance antitumor responses. One commonly used therapy is the application of IL-2, which can mediate a wide range of immune effects such as stimulating and activating T and NK cells and increasing T cell infiltration into tumors [13, 14]. Accumulative evidence indicates that the use of other costimulatory molecules, such as the tumor necrosis factor receptor (TNFR) family, can also enhance and augment the immunogenicity of the tumor. It has been demonstrated that engagement with the OX40 ligand (OX40L) or anti-OX40 mAb delivers signals to $OX40^+$ T cells, prolonging and propagating T cell responses [15]. Moreover, antibodies against OX40 rescue effector T cells from activation-induced cell death [16] and enhance antitumor immune responses *in vivo* [17, 18].

We have previously identified two HLA-A2.1/neu-restricted immunodominant epitopes (p369–377 and p773–782) [19]. To evaluate peptides specific for these epitopes, neu mice were crossed with A2.1/Kb transgenic mice [20] (A2×neu), and tetramer binding and cytotoxic activity were evaluated. The results indicate that A2×neu mice are functionally tolerant to neu; however, they contain a T cell repertoire of low avidity when compared to the repertoire of A2×FVB mice (FVB mice crossed with the A2.1/Kb mice). On the basis that a low-avidity repertoire persists in A2×neu mice, we examined the antitumor potential of these cells. Our results show that only multiple immunizations with pulsed DC in the presence of IL-2 induce an antitumor response which delays tumor growth in A2×neu mice. Furthermore, A2×neu mice immunized with DC in the presence of anti-OX40 mAb induce an antitumor response resulting in substantial tumor reduction. This enhancement was due to a greater number of expanded p369–377- and p773–782-specific $CD8^+$ T cells. Taken together, these results indicate that the low-avidity T cell repertoire for a self tumor antigen is functional, can be activated and expanded with the help of costimulation, and that these cells have the potential to provide a significant therapeutic benefit.

2 Results

2.1 Comparison of A2-neu-specific CTL responses between A2×neu and A2×FVB mice

Although previous reports have suggested that neu mice are tolerant to neu antigens [21–23], there are no available data on the evaluation of specific T cell responses. neu and FVB mice were crossed with A2.1/Kb transgenic mice, and T cell responses against the A2.1/neu p369–377 or p773–782 peptides were evaluated. Both the A2×neu and A2×FVB mice were immunized with DC pulsed with these peptides. Spleen cells were stimulated *in vitro* and analyzed for their ability to bind the A2.1-p369–377-PE and A2.1-p773–782-PE tetramers. As shown in Fig. 1A and B, $CD8^+$ T cells from A2×FVB mice bound the tetramers with higher intensity compared to $CD8^+$ T cells from A2×neu mice. The tetramers do not stain the HIV-POL CTL and no A2.1-p773–782-PE tetramer binding is detected in animals immunized with the p369–377 peptide or vice versa, demonstrating that binding to the tetramer is specific.

Stimulated spleen cells were analyzed for their lytic activity in a peptide dose curve assay. Compared to CTL from A2×FVB mice, CTL obtained from A2×neu mice demonstrated significantly lower recognition of both the p369–377 (Fig. 1C) and p773–782 (Fig. 1D) peptides. As a control for specificity, we used a restricted HLA-A2.1/HIV-POL CTL line [19], demonstrating that recognition of the neu-restricted CTL is specific. Taken together, these results strongly indicate that T cells from A2×neu mice are hyporesponsive to neu antigens.

2.2 CTL from A2×neu mice are of low avidity

The preceding results demonstrated that after immunization, the cytotoxic activity of the $CD8^+$ T cells from A2×FVB mice was superior to the cytotoxic activity of $CD8^+$ T cells from A2×neu mice. This raises the question whether the lack of an optimal response in A2×neu mice to immunodominant peptides was due to a quantity response (not enough p369–377 and p773–782 T cells existing in the repertoire) or a quality response (p369–377 and p773–782 CTL from A2×neu mice do not effectively recognize immunodominant peptides). Tetramer binding and cytotoxic activity were analyzed for p773–782 CTL lines derived from A2×neu and A2×FVB mice. The p773–782 CTL were stained with serial dilutions of the A2.1-p773–782-PE tetramer (1:50, 1:250 and 1:2,500). As shown in Fig. 2A, p773–782 CTL from A2×FVB mice demonstrate a stronger binding activity, while a weaker binding activity is detected in p773–782 CTL from A2×neu mice. We also analyzed TCR and CD8 expression of these CTL (Fig. 2B), and FACS analysis confirmed that CTL from both A2×neu and A2×FVB mice have the same level of expression of these molecules. These results suggest that the lower response of CTL from A2×neu mice could not be attributed to low expression of TCR and CD8 molecules. The CTL lines from A2×FVB mice showed a higher cytotoxic activity than CTL from A2×neu mice (Fig. 2C). Taken together, tetramer binding and cytotoxic activity show that there is a correlation, demonstrating a difference in the T cell avid-

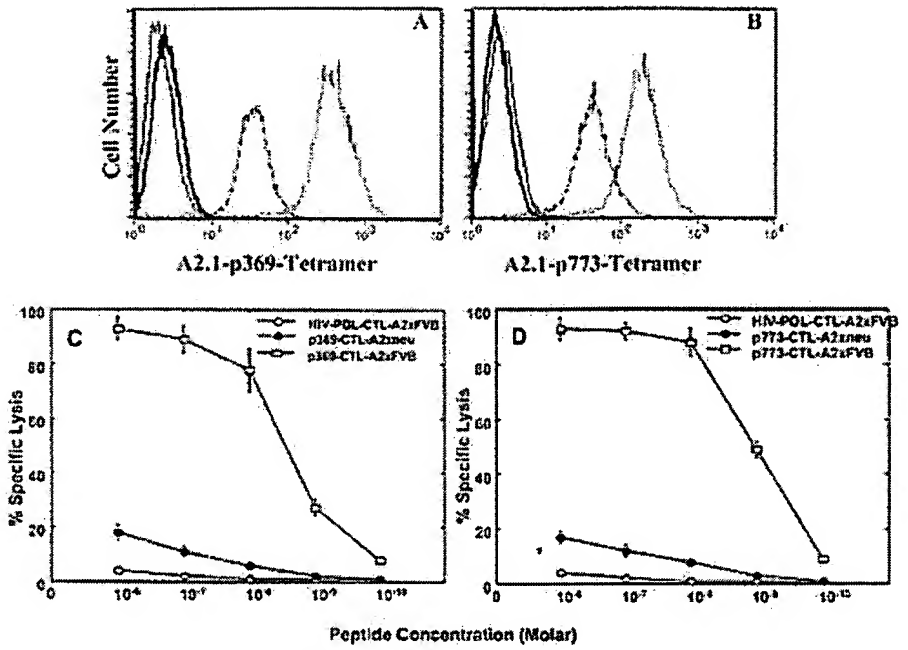

Fig. 1. The T cell repertoire of A2×neu mice is hyporesponsive to immunodominant epitopes. A2×neu and A2×FVB mice were immunized with DC pulsed with the p369–377 and p773–783 peptides, and restimulated T cells were stained with the A2.1-p369–377-PE and A2.1-p773–782-PE tetramers and anti-CD8-FITC. Viable $CD8^+$ cells were gated, and the fraction of cells stained with tetramers was analyzed. (A) Staining with A2.1-p369–377-PE tetramer. Thin line; p773–782 CTL. Thick line; HIV-POL CTL. Broken line; p369–377 CTL from A2×neu mice. Dotted line; p369–377 CTL from A2×FVB mice. (B) Staining with A2.1-p773–782-PE tetramer. Thin line; p369–377 CTL. Thick line; HIV-POL CTL. Broken line; p773–782 CTL from A2×neu mice. Dotted line; p773–782 CTL from A2×FVB mice. Lytic activity of spleen cells from p369–377 (C) and p773–782 (D) peptide-immunized animals. Stimulated spleen cells were assayed at an E:T ratio of 10:1 for cytotoxicity against T2-A2/Kb target cells pulsed with their respective peptides using decreasing concentrations of the peptides. The A2.1-HIV-POL-restriceted CTL were used as a control. Data are the means of four individually analyzed mice per group ± SD. The results shown are representative of three independent experiments.

ity between A2×neu and A2×FVB mice for the recognition of neu antigens, and indicating that A2×neu mice contain only low-avidity T cells for neu antigens.

2.3 CTL from A2×neu mice recognize neu antigens on tumors cells

We tested the ability of the p369–377- and p773–782-restricted CTL from both A2×neu and A2×FVB mice to recognize and kill tumors expressing HLA-A2.1 and neu molecules. For these experiments, we established a cell line (N202.A2) generated from a spontaneous tumor in A2×neu mice. The N202.A2 cells expressed A2.1 and neu molecules (Fig. 3A). As a control, we used the N202 cells, a cell line generated from a spontaneous tumor from neu mice [24] that does not express A2.1 molecules (Fig. 3A). CTL from A2×neu mice were capable of recognizing N202.A2 targets, albeit at significantly lower levels than CTL from A2×FVB mice (Fig. 3B). The CTL did not recognize N202 cells, indicating that they recognized A2-neu-restricted antigens expressed on tumor cells.

2.4 Adoptive transfer of low-avidity T cells delays tumor growth

Having demonstrated that CTL from A2×neu recognize the N202.A2 cells, we wanted to evaluate whether these CTL would have an antitumor effect. We confirmed that N202.A2 cells formed tumors in A2×neu mice but not in A2×FVB mice (data not shown). We analyzed the ability of A2×neu-derived $CD8^+$ T cells to reject N202.A2 cells after adoptive transfer. A critical question was how to maximize the antitumor activity of the low-avidity T cells. To this end, we tested whether multiple transfers would enhance the antitumor immune response. We compared animals that had received one, two or three transfers. A

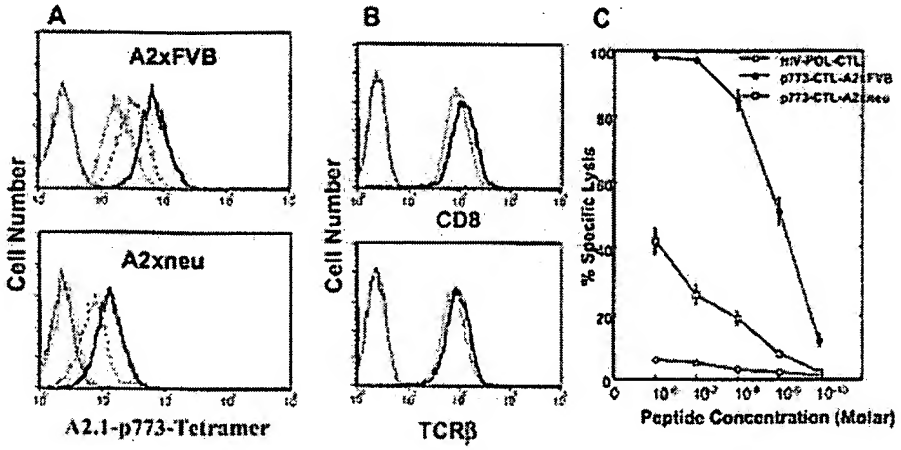

Fig. 2. CTL from A2×neu mice have low-avidity TCR. p773–782 CTL lines were established from A2×FVB and A2×neu mice. (A) CTL were stained with serial dilutions of A2.1-p773–783-PE tetramer. 1:50 thick line (2 μg); 1:250 dotted line; 1:2500 broken line; and the solid thin line shows cells stained with A2.1-p369–377-PE tetramer (2 μg) as a control. (B) CTL were analyzed for the surface expression of CD8 and TCR molecules. The thick line corresponds to CTL derived from A2×FVB mice, the broken line corresponds to CTL derived from A2×neu mice, and the thin line corresponds to unstained control. (C) Cytotoxic activity of CTL was analyzed against T2-A2.1/Kb target cells pulsed with decreasing concentrations of p773–782 peptide at an E:T ratio of 10:1. A2.1-HIV-POL-restricted CTL were used as a control. Similar results were found with p369–377 CTL (data not shown). Data presented are the means of two independent experiments ± SD.

single transfer of CTL derived from A2×FVB mice rejected the tumor (Fig. 4A). Interestingly, three transfers of the p369–377 (Fig. 4B) or p773–782 CTL (Fig. 4C) inhibited 40–45% of tumor growth, while fewer transfers demonstrated a lower efficiency for tumor growth inhibition (two transfers inhibited 25–30% and a single transfer inhibited 10–15% of tumor growth). These results demonstrate that the low-avidity CTL could delay tumor growth, indicating that these cells contribute to the antitumor response.

2.5 Immunization of A2×neu mice induces an antitumor response

The preceding results demonstrate that CTL derived from A2×neu mice have the capacity to recognize and kill the N202.A2 tumor cells *in vitro* and *in vivo*. Next, we evaluated whether immunization of A2×neu mice would induce an immune response capable of delaying growth or rejection of an established tumor. We compared animals that were immunized once, twice or three times with DC pulsed with p369–377 or p773–782 peptides in the presence of IL-2. Animals immunized three times with DC pulsed with p369–377 (Fig. 5A) or p773–782 (Fig. 5B) peptide showed ~25–30% tumor growth inhibition, while two immunizations induced ~12–15% tumor growth inhibition and one injection resulted in ~7–9% tumor growth inhibition (data not shown). We confirmed that the antitumor responses were $CD8^+$ T cell dependent since treatment with anti-CD8 mAb abrogated the antitumor response. These results suggest that, although the repertoire of neu mice is of low affinity, this repertoire can be activated and has antitumor potential.

2.6 Anti-OX40 mAb enhances the antitumor response in A2×neu mice

In the last few years, OX40 has gained importance as a costimulatory molecule capable of expanding immune responses and enhancing the antitumor immune responses of animals with established tumors [17, 18]. We evaluated whether the combination of DC-based vaccine and anti-OX40 mAb would stimulate a stronger antitumor response. As shown in Fig. 6, DC-based vaccines in the presence of anti-OX40 mAb induced a significantly stronger protective antitumor response resulting in ~45–50% tumor growth inhibition, while DC-based immunization in the absence of anti-OX40 mAb only inhibited ~25% of tumor growth. A weaker response was observed in animals that received either one or two immunizations with pulsed DC in the presence of IL-2 and anti-OX40 mAb (data not shown). Mice immunized three times with DC pulsed with HLA-A2.1/HIV-POL peptide in the presence of IL-2/anti-OX40 mAb showed minimal protection (~7% tumor growth inhibition), indicating the specificity of the antitumor responses induced by the

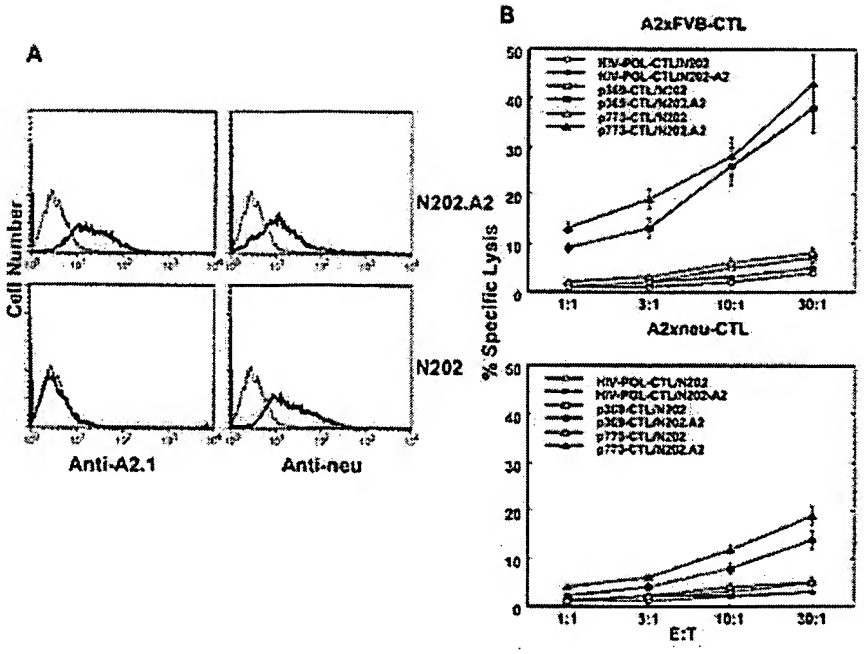

Fig. 3. Lysis of N202.A2 cells by p369–377 and p773–782 CTL. (A) N202.A2 and N202 cells were analyzed for the expression of HLA-A2.1 and neu. (B) The p369–377 and p773–782 CTL from A2×FVB and A2×neu mice were assayed for the specific killing activity of ^{51}Cr-labeled N202.A2 and N202 cells. Data presented are the means of three independent experiments ± SD.

p369–377 (Fig. 6A) or p773–782 (Fig. 6B) peptides. These results demonstrate that it is possible to manipulate the immune response in A2×neu mice and to considerably enhance the antitumor responses in tolerant hosts.

To evaluate the effect of anti-OX40 mAb on the antitumor response, we tested both tetramer binding and cytotoxic activity. As shown in Table 1, A2×neu mice immunized with DC pulsed with peptides in the presence of anti-OX40 mAb showed a higher number of spleen cells that bind the A2.1-p369–377-PE or A2.1-p773–782-PE tetra-

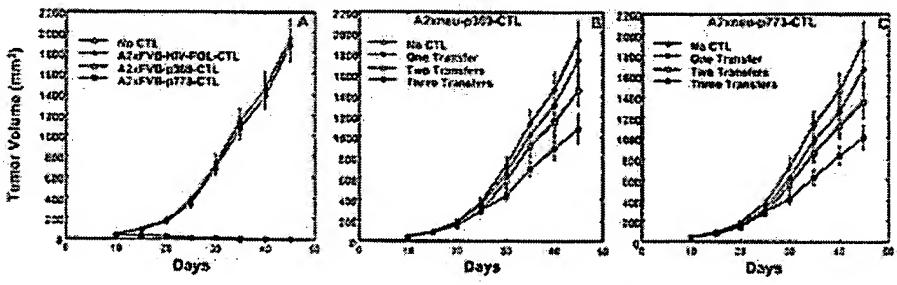

Fig. 4. Adoptive transfer of neu-reactive CTL from A2×neu mice delays growth of established tumors. N202.A2 cells (10^6) were injected s.c. in A2×neu mice on day 0. Animals received one (day 7), two (days 7 and 17) and three (days 7, 17 and 27) intratumoral transfers of *in vitro*-activated CTL. Each transfer consisted of 10^7 CTL. Animals were supplemented with daily i.p. injections of IL-2 (10^4 IU/day) for 1 week after each transfer. (A) Animals were transferred once with p369–377 or p773–782 CTL derived from A2×FVB mice. As a control for specificity, animals were transferred three times with the A2.1-HIV-POL-restriceted CTL derived from A2×FVB mice. (B) Animals were transferred one, two or three times with p369–377 CTL derived from A2×neu mice. (C) Animals were transferred one, two or three times with p773–782 CTL derived from A2×neu mice. Data are the means of two independent experiments ± SEM with at least five animals per group per experiment. A significant ($p<0.05$, Student's *t*-test) difference was found between mice that had received no CTL and mice that had received three transfers.

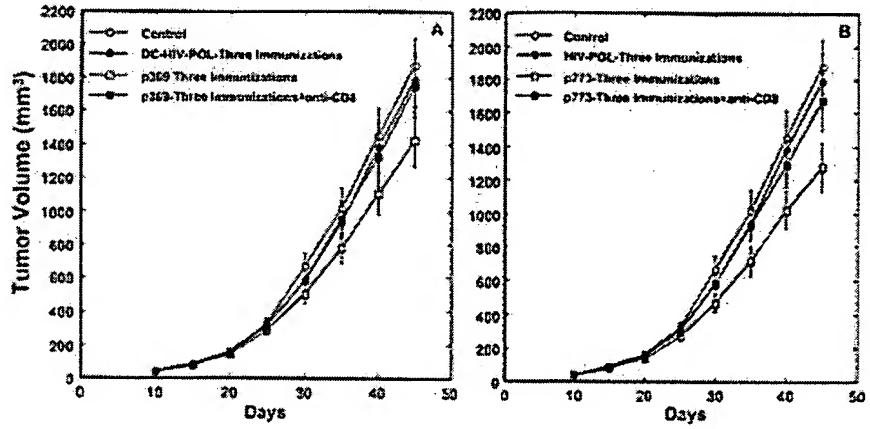

Fig. 5. Multiple immunizations with DC induce an antitumor response in A2×neu mice. A2×neu mice were inoculated s.c. on day 0 with 10^6 N202.A2 cells, and 1 week later animals were immunized as follows: Animals received one (on day 7), two (on days 7 and 17) and three (on days 7, 17 and 27) immunizations. Each immunization consisted of an s.c. injection of 10^6 DC pulsed with the peptides. Animals were supplemented with daily i.p. injections of IL-2 (10^4 IU/day) for 1 week after each immunization. (A) Animals were immunized one, two or three times with DC pulsed with the p369–377 peptide in the presence of IL-2. As a control, a group of animals was immunized three times with 10^6 DC pulsed with A2.1-HIV-POL peptide in the presence of IL-2. (B) Animals were immunized one, two or three times with DC pulsed with the p773–782 peptide in the presence of IL-2. Data are the means of two independent experiments ± SEM with at least five animals per group per experiment. A significant ($p<0.25$, Student's *t*-test) difference was found between the control group and mice that had received three immunizations.

mer as compared to animals without anti-OX40 mAb. We observed that immunization in the presence of IL-2, in contrast to anti-OX40 mAb, did not significantly augment the number of tetramer-binding cells. The p369–377 and p773–82 peptide-specific cytotoxic T cell responses were also augmented in animals treated with anti-OX40 mAb but minimally increased with IL-2 (Table 1).

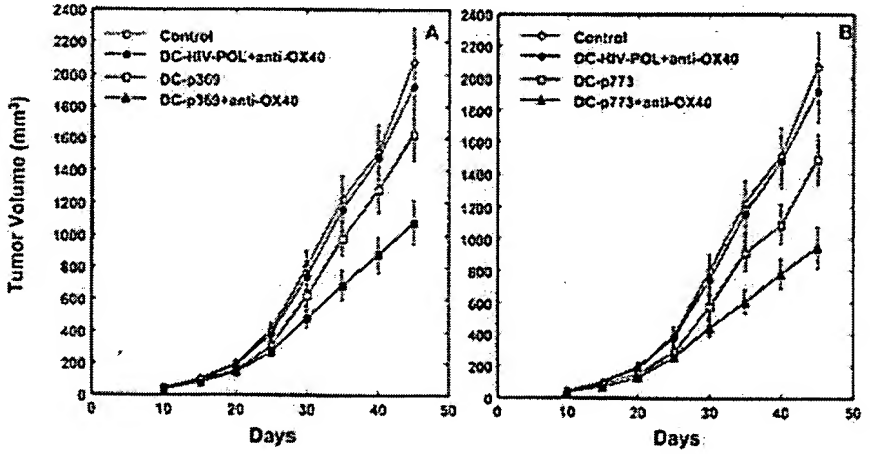

Fig. 6. Anti-OX40 enhances the antitumor response in A2×neu mice. A2×neu mice were inoculated s.c. on day 0 with 10^6 N202.A2 cells. Animals were immunized three times (on days 7, 17 and 27) with s.c. injections of 10^5 DC pulsed with the peptides. Animals were supplemented with daily i.p. injections of IL-2 (10^4 IU/day) for 1 week after each immunization. Anti-OX40 (100 µg/injection) was administered 2 days after each immunization. Data are the means of six to eight animals per group ± SEM. A significant ($p<0.01$, Student's *t*-test) difference was found between the control group and mice that had received three immunizations in the presence of anti-OX40 mAb.

Table 1. Effect of costimulatory molecules on the immune response of A2×neu mice

Immunization[a]	% Tetramer/CD8[b]		Cytotoxic activity[c]	
	p369–377 CTL	p773–782 CTL	p369–377 CTL	p773–782 CTL
DC-pep	1.2±0.2	2.1±0.3	12±3	15±2
DC-pep+IL-2	1.7±0.4	2.9±0.5	19±4	23±3
DC-pep+anti-OX40	4.4±0.7	6.8±0.9	34±4	42±6
DC-pep+IL-2+anti-OX40	4.8±0.8	7.1±1.2	37±6	45±5

[a] A2×neu mice (n=3–5 mice/group) were immunized with DC pulsed with p369–377 or p773–782 peptides in the presence or absence of IL-2 (administered daily 10^4 IU/day i.p. for 1 week starting 1 day after immunization) and anti-OX40 mAb (single injection of 100 μg starting 2 days after immunization).

[b] Ten days after immunization, animals were killed and spleen cells were stained with the A2.1-p369–377-PE or A2.1-p773–782-PE tetramer and anti-CD8-FITC Ab. Results are expressed as % (± SD) of tetramer-binding/CD8$^+$ cells for the mean of individual mice analyzed.

[c] Portion of the same spleens used for tetramer staining were restimulated *in vitro* and cells were tested for cytotoxicity against ^{51}Cr-labeled T2-A2.1/Kb target cells pulsed with p369–377 or p773–782 peptides at an E:T ratio of 10:1. Results are expressed as % of specific lysis ± SD for the mean of individual mice analyzed.

3 Discussion

The majority of TAA are self antigens and, therefore, T cell tolerance handicaps the immune response against such antigens. Understanding the behavior of T cell responses against self tumor antigens is of great importance for the development of effective immunotherapeutic strategies toward a treatment of cancer. To assess the effect of tolerance against neu responses, T cell responses against the p369–377 and p773–782 A2.1-neu-restricted peptides were evaluated in A2×neu mice. The analysis of the T cell responses demonstrates that A2×neu CD8$^+$ T cells bind with lower efficiency and intensity to the A2.1-p369–377-PE or A2.1-p773–782-PE tetramer, when compared to A2×FVB derived CD8$^+$ T cells, and that A2×neu CD8$^+$ T cells required 10–100 times more peptide to achieve comparable lysis than T cells from A2×FVB mice. The lower response of the CTL from A2×neu mice could not be attributed to low expression of TCR and CD8 molecules, indicating that the TCR on these CTL are of low affinity. Taken together, these results demonstrate that A2×neu mice are tolerant to neu antigens when compared to A2×FVB mice, and tolerance is achieved by the elimination of T cells with high avidity [25].

The low-avidity T cells that persist in A2×neu mice were able to recognize and kill tumor cells expressing HLA-2.1/neu antigens. We wanted to determine the antitumor potential of these cells. Previous studies have shown that plasmid DNA vaccination [26] or allogeneic cell vaccination [21] could induce a protective immune response in neu mice. In contrast to these studies, in which animals were immunized first and then challenged with the tumor, we used a therapeutic tumor model whereby animals were first implanted with the tumor and then immunized. Our results demonstrate that, to generate a protective immunity in neu mice, the application of multiple immunizations is critical. In the adoptive transfer experiments, a single T cell transfer has a minimal effect on tumor growth; however, sequential adoptive transfers of T cells significantly delayed tumor growth. Similar results were found with immunization of DC pulsed with the p369–377 and p773–782 peptides. Single immunization inhibited 7% of tumor growth while three immunizations inhibited tumor growth by 25–30%. The application of multiple immunizations could have important implications for the design of immunotherapeutic strategies targeting self tumor antigens. The significance of applying multiple immunizations could be interpreted as follows: (1) it might be necessary to constantly restimulate the already activated immune response with professional APC, since the interaction of the low-avidity T cells and tumor cells might not be sufficient to actively maintain the effector function of tumor-specific T cells; (2) it might be possible that the stimulated tumor-specific T cells are further tolerized or deleted after initial interaction with the tumor, sequential immunizations could then activate new responses; or (3) as demonstrated by Cordaro et al. [27], the T cell response is capable of inducing an effective antitumor response only when a memory low-avidity T cell response is generated. It might be that the application of multiple immunizations in A2×neu mice generates a memory response, resulting in the most efficient strategy to induce tumor immunity in these mice.

Recently, Ercolini et al. [28] reported the identification of an H2D^q-neu-restricted epitope. Two immunizations with

DC pulsed with this peptide prior to tumor challenge did not induce a protective immunity in neu mice. We have also observed a minimal protection (less than 10% of tumor growth inhibition) in animals that were immunized three times with DC pulsed with p369–377 or p773–782 peptides in the absence of IL-2 (data not shown). De Visser et al. [29] demonstrated that levels of IL-2 were markedly reduced in low-avidity T cells and that the effector function of these cells was severely impaired. Our data were in agreement with their results in that the induction of an antitumor response was dependent on the addition of IL-2. Interestingly, in contrast to anti-OX40 mAb, the addition of IL-2 did not significantly augment the number of tetramer-binding cells after DC immunization. The exact role of IL-2 in this model remains to be determined. The role of IL-2 could be to maintain the effector function of the T cell response [13, 14]. However, DC immunization in combination with anti-OX40 mAb has a synergistic effect, resulting in substantial tumor growth inhibition. Even though previous studies have demonstrated that injections of anti-OX40 mAb are sufficient to induce the eradication of established tumors [17, 18], the therapeutic effect of anti-OX40 mAb seems to be limited in the neu tumor model. This could be because of the low immunogenicity of the tumor in these mice. We do not yet know for certain how anti-OX40 mAb influences the immune responses. Anti-OX40 mAb might stimulate $CD4^+$ T cells [15, 16] to provide help to $CD8^+$ T cells and, as a consequence, stronger antitumor responses can be observed. The other mechanism in which anti-OX40 could exert its effect is by directly enhancing the $CD8^+$ T cell responses, as demonstrated by De Smedt et al. [30]. The tetramer-binding and cytotoxic assays indicate that $CD8^+$ T cell responses in A2×neu mice are enhanced in the presence of anti-OX40 mAb, and the increased presence of tetramer-binding cells correlates with the cytotoxic activity. These data suggest that the improvement of the immune response in the presence of anti-OX40 is most probably due to a greater number of tumor-specific T cells that were expanded after immunization with DC. Taken together, these data indicate that for the activation of an immune response in A2×neu mice, the use of costimulatory molecules such as IL-2 or anti-OX40 mAb might be critical to optimally stimulate/expand the low-avidity repertoire in order to generate antitumor immunity.

With the use of the A2×neu mice, we have established that tolerance in neu mice is a consequence of the deletion of high-avidity T cells. Although most of the effective treatments correlate with immune responses that are of high avidity, the observation that low-avidity T cells can recognize and kill tumor cells [9, 10, 27, 29] offers an opportunity to exploit these cells for the induction of antitumor responses. It is highly probable that immunotherapy alone under tolerant conditions will not be sufficient to induce the complete eradication of tumors. However, if the immune repertoire for a self tumor antigen is properly stimulated and used in combination with another form of therapy it could result in rejection of the tumor. In this regard, we have recently evaluated the combination of immunotherapy and antiangiogenic therapy. Our results demonstrate that the application of each therapy alone retarded tumor growth; however, when these therapies were combined they resulted in complete rejection of the tumor in neu mice [31]. These results further confirmed the antitumor potential of low-avidity T cells and the utility of this repertoire in antitumor responses. In these studies, we demonstrated that if the immune responses from tolerant hosts were properly primed and the effector function was maintained over time, the immune response would contribute to the destruction of the tumor. Further evaluations of the low-avidity repertoire against self tumor antigens will ultimately lead to improvements of the strategies for the optimization of vaccination immunotherapies against self tumor antigens.

4 Materials and methods

4.1 Mice and cell lines

The neu transgenic mice (line 202) were commercially obtained from Jackson Laboratories (Bar Harbor, ME) and maintained homozygously. The FVB mice were purchased from Harlan (Indianapolis, IN). The HLA-A2.1/Kb transgenic mice were kindly provided by Dr. Linda Sherman from the Scripps Research Institute (La Jolla, CA). The neu and FVB mice were mated with the HLA-A2.1/Kb mice to generate A2×neu and A2×FVB mice. The N202.A2 cell line was established from a spontaneous tumor. The tumor was digested with a mixture of 1 mg/ml collagenase, 10 U/ml hyaluronidase and 20 μg/ml DNase (all from Sigma, St. Louis, MO). Cells were grown in RPMI 1640 medium supplemented with 20% FCS, 2 mM L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol (2-ME) and 50 μg/ml gentamicin. Cultured cells were treated by differential trypsinization to remove fibroblasts. The N202 mammary cell line was obtained from Dr. Pier-Luigi Lollini (University of Bologna, Bologna, Italy). The T2-A2.1/Kb cell line was provided by Dr. Linda Sherman. Anti-OX40 (OX86) mAb were obtained from the European Cell Culture Collection (Wiltshire, GB). All cell lines were maintained in complete RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 5×10^{-5} M 2-ME and 50 μg/ml gentamicin.

4.2 Flow cytometry analysis

The N202.A2 and N202 cells were stained with primary antibodies (0.5 μg/ml) against the human HLA-A2 antigen

(PA2.1), murine $H2D^d/H\text{-}2L^d$ (KH117; PharMingen, San Diego, CA) and rat neu (neu-Ab-4; Calbiochem, San Diego, CA). To measure the expression of CD8 and TCRβ molecules on CTL, the anti-CD8α-FITC (53-6.7; PharMingen) and anti-TCRβ-FITC (H57–597; PharMingen) were used, respectively. Samples were analyzed in a FACScan (Becton Dickinson, San Diego, CA) and data analysis was performed using CellQuest software.

4.3 Generation of DC and immunization of A2×neu and A2×FVB mice

For DC isolation, magnetic beads conjugated with anti-CD4, anti-CD8 and anti-B220 (Dynal, Oslo, Norway) were used to deplete bone marrow cells of lymphocytes. The remaining cells were cultured in complete RPMI medium containing 3% GM-CSF (supernatant from J558L cells transfected with the murine GM-CSF gene obtained from Dr. R Steinman, Rockefeller University, New York, NY). For DC maturation, 100 U/ml TNF-α was added on day 7 and DC were collected on day 9. Maturation of pulsed DC was confirmed by evaluating the expression of B7-1, B7-2, MHC class I and MHC class II. Mature DC were pulsed with the p369–377 or p773–782 peptides at 10 μg/ml for 3 h at 37°C.

4.4 Stimulation of p369–377- and p773–782-A2.1-neu-restricted CTL responses

Ten days after immunization with DC, animals were killed and spleens were removed. For stimulation of cultures, T cells (10^6 cells/well) were incubated with autologous irradiated (3,000 rad) LPS spleen blasts (2×10^5 cells/well) that were pulsed with the p369–377 (KIFGSLAFL) and p773–782 (VMAGVGSPYV) peptides in 24-well plates. After 5 days, CTL were assayed for lytic activity. The N202.A2, N202 and T2-A2.1/Kb cells pulsed with the peptides were incubated with 150 μCi sodium [^{51}Cr]chromate for 1 h at 37°C. Cells were washed three times and resuspended in complete RPMI medium. For the cytotoxic assay, ^{51}Cr-labeled target cells (10^4) were incubated with varying concentrations of effector cells in a final volume of 200 μl in U-bottom 96-well microtiter plates. Supernatants were recovered after 6 h of incubation.

4.5 Tetramer staining

The A2.1-p369-377-PE and A2.1-p773-782-PE tetramers were obtained from the NIAID Tetramer Core Facility. Spleen cells from mice immunized with the p369-377 and p773-782 peptides were stained with the A2.1-p369-377-PE and A2.1-p773-782-PE tetramers (2 μg/sample) for 1 h at room temperature and then with anti-CD8-FITC for an additional 30 min at 4°C. Samples were analyze in a FACScan.

4.6 Adoptive Transfer

The CTL lines specific for p369–377 and p773–782 peptides derived from A2×neu or A2×FVB mice were expanded *in vitro* by mixing 2×10^5 cultured T cells with 2×10^6 irradiated autologous LPS spleen blast cells pulsed with the p369–377 or p773–782 peptides containing 25 U/ml IL-2 in T25 flasks in a total volume of 10 ml. CTL were tested for cytolytic activity after *in vitro* stimulation. To test the effect of adoptively transferred T cells, A2×neu mice were implanted s.c. with 10^6 N202.A2 tumor cells. On day 7 after tumor inoculation, animals were randomly divided into groups of five, and 10^7 stimulated CTL were directly injected into the tumor. Animals received daily i.p. injections of IL-2 for 1 week (10^4 IU/day). Tumor growth was monitored every 5 days and growth rates were determined by caliper measurements in two diameters. Tumor volume was expressed as: (minor diameter)^2×major diameter/2. Statistical analysis was determined by Student's *t*-test.

4.7 Immunization

A2×neu mice were implanted s.c. with 10^6 N202.A2 cells on day 0. On day 7, animals were randomly divided into groups of five and immunized once, twice or three times s.c. with 10^6 DC pulsed with the p369–377 or p773–782 peptides in the presence or absence of rIL-2. Animals that received rIL-2 were injected with 10^4 IU/day for different periods of time as described in the experiments. Anti-OX40 (100 μg/injection) was injected 2 days after DC immunization and once a week thereafter.

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References

1 **Lurquin, C., Van Pel, A., Mariamo, B., De Plaen, E., Szikora, J. P., Janssens, C., Reddehase, M. J., Lejeune, J. and Boon, T.,** Structure of the gene of tum$^-$ transplantation antigen P91A: the mutated exon encodes a peptide recognized with Ld by cytolytic T cells. *Cell* 1989. 58: 293–303.

2 **van der Bruggen, P., Traversari, C., Chomez, P., Lurquin, C., De Plaen, E., Van den Eynde, B., Knuth, A. and Boon, T.,** A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991. 254: 1643–1647.

3 **Valmori, D., Levy, F., Miconnet, I., Zajac, P., Spagnoli, G. C., Rimoldi, D., Lienard, D., Cerundolo, V., Cerottini, J. C. and Romero, P.,** Induction of potent antitumor CTL responses by recombinant vaccinia encoding a melan-A peptide analogue. *J. Immunol.* 2000. 164: 1125–1131.

4 **Rosenberg, S. A.,** A new era for cancer immunotherapy based on the genes that encode cancer antigens. *Immunity* 1999. 10: 281–287.

5 **Levitsky, H. I.**, Augmentation of host immune responses to cancer: overcoming the barrier of tumor antigen-specific T cell tolerance. *Cancer J.* 2000. **6 Suppl. 3**: S281–290.

6 **Ferber, I., Schonrich, G., Schenkel, J., Mellor, A. L., Hammerling, G. J. and Arnold, B.**, Levels of peripheral T cell tolerance induced by different doses of tolerogen. *Science* 1994. **263**: 674–676.

7 **Kawai, K. and Ohashi, P. S.**, Immunological function of a defined T cell population tolerized to low-affinity self antigens. *Nature* 1995. **374**: 68–69.

8 **Hausmann, S., Martin, M., Gauthier, L. and Wucherpfennig, K. W.**, Structural features of autoreactive TCR that determine the degree of degeneracy in peptide recognition. *J. Immunol.* 1999. **162**: 338–344.

9 **Morgan, D. J., Kreuwel, H. T., Fleck, S., Levitsky, H. I., Pardoll, D. M. and Sherman, L. A.**, Activation of low avidity CTL specific for a self epitope results in tumor rejection but not autoimmunity. *J. Immunol.* 1998. **160**: 643–651.

10 **Mullins, D. W., Bullock, T. N., Colella, T. A., Robila, V. V. and Engelhard, V. H.**, Immune responses to the HLA-A*0201-restricted epitopes of tyrosinase and glycoprotein 100 enable control of melanoma outgrowth in HLA A*0201-transgenic mice. *J. Immunol.* 2001. **167**: 4853–4860.

11 **Muller, W. J., Sinn, E., Pattengale, P. K., Wallace, R. and Leder, P.**, Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell* 1988. **54**: 105–115.

12 **Guy, C. T., Webster, M. A., Schaller, M., Parsons, T. J., Cardiff, R. D. and Muller, W. J.**, Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc. Natl. Acad. Sci. USA* 1992. **89**: 10578–10582.

13 **Shimizu, K., Fields, R. C., Giedlin, M. and Mule, J. J.**, Systemic administration of interleukin 2 enhances the therapeutic efficacy of dendritic cell-based tumor vaccines. *Proc. Natl. Acad. Sci. USA* 1999. **96**: 2268–2273.

14 **Dai, Z., Konieczny, B. T. and Lakkis, F. G.**, The dual role of IL-2 in the generation and maintenance of CD8$^+$ memory T cells. *J. Immunol.* 2000. **165**: 3031–3036.

15 **Gramaglia, I., Weinberg, A. D., Lemon, M. and Croft, M.**, Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. *J. Immunol.* 1998. **161**: 6510–6517.

16 **Rogers, P. R., Song, J., Gramaglia, I., Killeen, N. and Croft, M.**, OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells. *Immunity* 2001. **15**: 445–455.

17 **Weinberg, A. D., Rivera, M. M., Prell, R., Morris, A., Ramstad, T., Vetto, J. T., Urba, W. J., Alvord, G., Bunce, C. and Shields, J.**, Engagement of the OX-40 receptor in vivo enhances antitumor immunity. *J. Immunol.* 2000. **164**: 2160–2169.

18 **Pan, P. Y., Zang, Y., Weber, K., Meseck, M. L. and Chen, S. H.**, OX40 ligation enhances primary and memory cytotoxic T lymphocyte responses in an immunotherapy for hepatic colon metastases. *Mol. Ther.* 2002. **6**: 528–536.

19 **Lustgarten, J., Theobald, M., Labadie, C., LaFace, D., Peterson, P., Disis, M. L., Cheever, M. A. and Sherman, L. A.**, Identification of Her-2/Neu CTL epitopes using double transgenic mice expressing HLA-A2.1 and human CD8. *Hum. Immunol.* 1997. **52**: 109–118.

20 **Vitiello, A., Marchesini, D., Furze, J., Sherman, L. A. and Chesnut, R. W.**, Analysis of the HLA-restricted influenza-specific cytotoxic T lymphocyte response in transgenic mice carrying a chimeric human-mouse class I major histocompatibility complex. *J. Exp. Med.* 1991. **173**: 1007–1015.

21 **Reilly, R. T., Gottlieb, M. B., Ercolini, A. M., Machiels, J. P., Kane, C. E., Okoye, F. I., Muller, W. J., Dixon, K. H. and Jaffee, E. M.**, HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. *Cancer Res.* 2000. **60**: 3569–3576.

22 **Kurt, R. A., Whitaker, R., Baher, A., Seung, S. and Urba, W. J.**, Spontaneous mammary carcinomas fail to induce an immune response in syngeneic FVBN202 neu transgenic mice. *Int. J. Cancer* 2000. **87**: 688–694.

23 **Reilly, R. T., Machiels, J. P., Emens, L. A., Ercolini, A. M., Okoye, F. I., Lei, R. Y., Weintraub, D. and Jaffee, E. M.**, The collaboration of both humoral and cellular HER-2/neu-targeted immune responses is required for the complete eradication of HER-2/neu-expressing tumors. *Cancer Res.* 2001. **61**: 880–883.

24 **Lollini, P. L., Nicoletti, G., Landuzzi, L., De Giovanni, C., Rossi, I., Di Carlo, E., Musiani, P., Muller, W. J. and Nanni, P.**, Down regulation of major histocompatibility complex class I expression in mammary carcinoma of HER-2/neu transgenic mice. *Int. J. Cancer* 1998. **77**: 937–941.

25 **Theobald, M., Biggs, J., Hernandez, J., Lustgarten, J., Labadie, C. and Sherman, L. A.**, Tolerance to p53 by A2.1-restricted cytotoxic T lymphocytes. *J. Exp. Med.* 1997. **185**: 833–841.

26 **Chen, Y., Hu, D., Eling, D. J., Robbins, J. and Kipps, T. J.**, DNA vaccines encoding full-length or truncated Neu induce protective immunity against Neu-expressing mammary tumors. *Cancer Res.* 1998. **58**: 1965–1971.

27 **Cordaro, T. A., de Visser, K. E., Tirion, F. H., Schumacher, T. N. and Kruisbeek, A. M.**, Can the low-avidity self-specific T cell repertoire be exploited for tumor rejection? *J. Immunol.* 2002. **168**: 651–660.

28 **Ercolini, A. M., Machiels, J. P., Chen, Y. C., Slansky, J. E., Giedlen, M., Reilly, R. T. and Jaffee, E. M.**, Identification and characterization of the immunodominant rat HER-2/neu MHC cClass I epitope presented by spontaneous mammary tumors from HER-2/neu-transgenic mice. *J. Immunol.* 2003. **170**: 4273–4280.

29 **de Visser, K. E., Cordaro, T. A., Kioussis, D., Haanen, J. B., Schumacher, T. N. and Kruisbeek, A. M.**, Tracing and characterization of the low-avidity self-specific T cell repertoire. *Eur. J. Immunol.* 2000. **30**: 1458–1468.

30 **De Smedt, T., Smith, J., Baum, P., Fanslow, W., Butz, E. and Maliszewski, C.**, Ox40 costimulation enhances the development of T cell responses induced by dendritic cells *in vivo*. *J. Immunol.* 2002. **168**: 661–670.

31 **Cuadros, C. D. A., Frost, G., Borgstrom P. and Lustgarten, J.**, Cooperative effect between immunotherapy and antiangiogenic therapy results in effective tumor rejection in tolerant Her-2/neu mice. *Cancer Res.* 2003. **63**: 5895–5901.

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